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(74) Agents: **LARCHER, Carol** et al.; Leydig, Voit & Mayer,
Ltd., Suite 4900, Two Prudential Plaza, 180 North Stetson,
Chicago, IL 60601-6780 (US).

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(71) Applicant (*for all designated States except US*): **THE
GOVERNMENT OF THE UNITED STATES OF
AMERICA**, represented by **THE SECRETARY, DE-
PARTMENT OF HEALTH AND HUMAN SERVICES**
[US/US]; National Institutes of Health Office of Tech-
nology Transfer, Suite 325, 6011 Executive Boulevard,
Rockville, MD 20852 (US).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **DIMITROV,**
Dimitar, S. [US/US]; 12309 Braxfield Court, #16,
Rockville, MD 20852 (US). **ZHANG, Mei-Yun** [CN/US];
1122 Taney Avenue, Frederick, MD 21702 (US).

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(54) Title: IDENTIFICATION OF NOVEL BROADLY CROSS-REACTIVE HIV-1 NEUTRALIZING HUMAN MONOCLONAL ANTIBODIES

(57) Abstract: The present invention provides an antibody to human immunodeficiency virus (HIV) envelope glycoprotein that can recognize one or more strains of HIV, wherein the epitope of HIV recognized by the antibody is inducible, and wherein the antibody binding to the epitope is enhanced by the presence of CD4 and the HIV co-receptor, and related fusion proteins, conjugates, nucleic acids, vectors, host cells, compositions and methods of use to inhibit an infection of a human at risk of becoming infected with HIV, to reduce the severity of an infection of a human infected with HIV, and to treat an infection of a human with HIV.



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IDENTIFICATION OF NOVEL BROADLY CROSS-REACTIVE HIV-1 NEUTRALIZING HUMAN MONOCLONAL ANTIBODIES

TECHNICAL FIELD OF THE INVENTION

5 The present invention relates to antibodies, in particular monoclonal antibodies, specifically cross-reactive human immunodeficiency virus (HIV) neutralizing antibodies, and related fusion proteins, conjugates, nucleic acids, vectors, host cells, compositions, and methods of use.

10 BACKGROUND OF THE INVENTION

Binding of the HIV-1 envelope glycoprotein (Env, gp120-gp41) to CD4 and coreceptors initiates a series of conformational changes that are the heart of the fusion machinery leading to viral entry¹. The elucidation of the nature of the Env conformational changes is not only a clue to the mechanism of HIV type 1 (HIV-1) entry but may also provide new tools for the development of inhibitors and vaccines.^{2,3} It has been proposed that the interaction of coreceptor molecules with the Env-CD4 complex leads to intermediate Env conformations that may include structures conserved among various HIV-1 isolates that could be used as vaccines^{4,5}. Of the four known potent broadly neutralizing antibodies (b12⁷, 2F5^{8,9}, 2G12¹⁰, and 4E10/Z13¹¹), none has a receptor-inducible epitope.

No single broadly cross-reactive monoclonal antibody with potent neutralization activity for all primary HIV isolates has been isolated and characterized. Typically, monoclonal antibodies against CD4-inducible epitopes, such as 17b and CG10, are only weakly neutralizing against primary isolates¹⁶, suggesting that CD4-inducible epitopes on gp120 may not serve as targets for potent broadly neutralizing antibodies.

It is an object of the present invention to provide antibodies to receptor-inducible epitopes, wherein the antibodies exhibit high affinity of binding of HIV and neutralizing activity. The antibodies can be used, alone or in combination with other active agents or as fusion proteins or conjugates with other active agents, to inhibit HIV and as tools to dissect mechanisms of HIV cellular entry. This and other objects and advantages of the present invention, as well as additional inventive features, will become apparent from the detailed description provided herein.

BRIEF SUMMARY OF THE INVENTION

The present invention provides an isolated or purified nucleic acid molecule comprising the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 8 or a variant of any of the foregoing, wherein the
5 variant retains the ability to bind to the same epitope as that of SEQ ID NO: 1, 2 or 8 respectively, to a greater or lesser extent. Also provided are a vector comprising such a nucleic acid molecule, a composition comprising the nucleic acid molecule, optionally in the form of a vector, and a host cell comprising the nucleic acid molecule, optionally in the form of a vector.

10 The present invention further provides an antibody to HIV envelope glycoprotein that can recognize one or more strains of HIV, wherein the epitope of HIV recognized by the antibody is inducible, and wherein the antibody binding to the epitope is enhanced by the presence of CD4 and the HIV co-receptor. Also provided are a composition comprising the antibody, a fusion protein or conjugate comprising
15 the antibody, and a composition comprising the fusion protein or conjugate.

The present invention still further provides methods of using the above nucleic acid molecules, vectors, host cells, antibodies, fusion proteins and conjugates. In one embodiment, the present invention provides a method of inhibiting an infection of a human at risk of becoming infected with HIV. In another embodiment, the present
20 invention provides a method of reducing the severity of an infection of a human infected with HIV. In yet another embodiment, the present invention provides a method of treating an infection of a human with HIV. The methods comprise administering to the human an isolated or purified nucleic acid molecule encoding an above-described antibody; optionally as part of a fusion protein, wherein the nucleic
25 acid molecule is optionally in the form of a vector and/or optionally contained within a cell, or the antibody, itself, optionally as part of a fusion protein or conjugate.

BRIEF DESCRIPTION OF THE FIGURES

- Fig. 1 is a flow chart of sequential panning (m6 and m9).
30 Fig. 2 is the scFv amino acid sequence alignment, in which the mutated amino acids are indicated in bold (SEQ ID NOS: 1-3).
Fig. 3 is the amino acid sequence of the X5 light chain (SEQ ID NO: 4).

Fig. 4 is the amino acid sequence of the X5 heavy chain (SEQ ID NO: 5).

Fig. 5 is the DNA (SEQ ID NO: 6) and amino acid (SEQ ID NO: 7) sequences of domain 2 of CD4.

Fig. 6 is the amino acid sequence of an ScFv-CD4 fusion protein comprising
5 from the amino terminus to the carboxy terminus (read from left to right and top to bottom) m6, linker and domain 2 of CD4 (SEQ ID NO: 8).

DETAILED DESCRIPTION OF THE PRESENT INVENTION

The present invention may be understood more readily by reference to the
10 following detailed description of preferred embodiments of the invention and the Examples included therein and to the Figures and their previous and following description.

Before the present compounds, compositions, and/or methods are disclosed and described, it is to be understood that this invention is not limited to specific synthetic
15 methods, specific recombinant biotechnology methods unless otherwise specified, or to particular reagents, pharmaceutical formulations or administration regimens unless otherwise specified, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

20 It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" can include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a compound" includes mixtures of compounds, reference to "a pharmaceutical carrier" includes mixtures of two or more such carriers, and the like.

25 In this specification and in the claims which follow, reference will be made to a number of terms, which shall be defined to have the following meanings:

Herein the term "sequential antigen panning" refers to a method of producing an antibody or antibodies comprising isolating the antibody or antibodies by screening a phage display library for antibodies that can bind to an antigen, wherein the isolation is
30 continued by screening the binding antibodies for the ability to bind the antigen at a lower concentration or to bind an additional antigen, wherein this process can continue

for two or more cycles, wherein the antibody or antibodies that bind on the last cycle are selected.

Herein the term "screening" refers to a method of isolating an antibody or antibodies from other antibodies, based on the level of binding activity to an antigen.

5 An example of a screening method is a phage ELISA.

Herein the term "selecting" refers to a method of isolating an antibody or antibodies from other antibodies based on the ability to bind an antigen.

In view of the foregoing, the present invention provides an isolated or purified nucleic acid molecule comprising the nucleotide sequence encoding the amino acid
10 sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 8 or a variant of any of the foregoing, wherein the variant retains the ability to bind to the same epitope as that of SEQ ID NO: 1, 2 or 8, respectively, to a greater or lesser extent.

The term "isolated" as used herein means having been removed from its natural environment. The term "purified" as used herein means having been increased in
15 purity, wherein "purity" is a relative term, and not to be construed as absolute purity. The term "nucleic acid molecule" as used herein means a polymer of DNA or RNA, (i.e., a polynucleotide), which can be single-stranded or double-stranded, synthesized or obtained from natural sources, and which can contain natural, non-natural or altered nucleotides. Such nucleic acid molecules can be synthesized in accordance with
20 methods well-known in the art.

The nucleic acid molecule encoding a variant can comprise one or more mutations. By "mutation" is meant any insertion, deletion, substitution and/or inversion in a given oligonucleotide. Such mutated oligonucleotides and fragments thereof can be obtained from naturally occurring sources or generated using methods known in the
25 art. For instance, site-specific mutations can be introduced by ligating into an expression vector a synthesized oligonucleotide comprising the mutation(s). Alternately, oligonucleotide-directed site-specific mutagenesis procedures can be used, such as disclosed in Walder et al., Gene 42: 133 (1986); Bauer et al., Gene 37: 73 (1985); Craik, Biotechniques, 12-19 (January 1995); and U.S. Patent Nos. 4,518,584
30 and 4,737,462. A preferred means for introducing mutations is the QuikChange Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA). While the above-described mutated oligonucleotides and fragments thereof can be generated *in vivo* and then

isolated or purified, alternatively, they can be synthesized. A variety of techniques used to synthesize the oligonucleotides and fragments thereof of the present invention are known in the art. See, for example, Lemaitre et al., *Proceedings of the National Academy of the Sciences* 84: 648-652 (1987) and the references cited herein under

5 “EXAMPLES.” The oligonucleotides and fragments thereof of the present invention can alternatively be synthesized by companies, such as Eurogentec, Belgium. Preferably, the nucleotides encoding CDRH3 remain unchanged or are only slightly changed, such as by conservative or neutral amino acid substitution(s) (see, e.g., Fig. 2 and 3). Mutations can be tolerated elsewhere. Activity of the encoded antibody can be

10 assess *in vitro* under physiological conditions.

A vector comprising any of the above-described isolated or purified nucleic acid molecules, or fragments thereof, is further provided by the present invention. Any of the above nucleic acid molecules, or fragments thereof, can be cloned into any suitable vector and can be used to transform or transfect any suitable host. The selection of

15 vectors and methods to construct them are commonly known to persons of ordinary skill in the art and are described in general technical references (see, in general, “Recombinant DNA Part D,” *Methods in Enzymology*, Vol. 153, Wu and Grossman, eds., Academic Press (1987)). Desirably, the vector comprises regulatory sequences, such as transcription and translation initiation and termination codons, which are

20 specific to the type of host (e.g., bacterium, fungus, plant or animal) into which the vector is to be introduced, as appropriate and taking into consideration whether the vector is DNA or RNA. Preferably, the vector comprises regulatory sequences that are specific to the genus of the host. Most preferably, the vector comprises regulatory sequences that are specific to the species of the host.

25 Constructs of vectors, which are circular or linear, can be prepared to contain an entire nucleic acid sequence as described above or a portion thereof ligated to a replication system functional in a prokaryotic or eukaryotic host cell. Replication systems can be derived from ColE1, 2 μ plasmid, λ , SV40, bovine papilloma virus, and the like.

30 In addition to the replication system and the inserted nucleic acid, the construct can include one or more marker genes, which allow for selection of transformed or transfected hosts. Marker genes include biocide resistance, e.g., resistance to

antibiotics, heavy metals, etc., complementation in an auxotrophic host to provide prototrophy, and the like.

Suitable vectors include those designed for propagation and expansion or for expression or both. A preferred cloning vector is selected from the group consisting of the pUC series, the pBluescript series (Stratagene, LaJolla, CA), the pET series (Novagen, Madison, WI), the pGEX series (Pharmacia Biotech, Uppsala, Sweden), and the pEX series (Clontech, Palo Alto, CA). Bacteriophage vectors, such as λ GT10, λ GT11, λ ZapII (Stratagene), λ EMBL4, and λ NM1149, also can be used. Examples of plant expression vectors include pBI101, pBI101.2, pBI101.3, pBI121 and pBIN19 (Clontech). Examples of animal expression vectors include pEUK-C1, pMAM and pMAMneo (Clontech). The TOPO cloning system (Invitrogen, Carlsbad, CA) also can be used in accordance with the manufacturer's recommendations.

An expression vector can comprise a native or nonnative promoter operably linked to an isolated or purified nucleic acid molecule as described above. The selection of promoters, e.g., strong, weak, inducible, tissue-specific and developmental-specific, is within the skill in the art. Similarly, the combining of a nucleic acid molecule, or fragment thereof, as described above with a promoter is also within the skill in the art.

Suitable viral vectors include, for example, retroviral vectors, parvovirus-based vectors, e.g., adeno-associated virus (AAV)-based vectors, AAV-adenoviral chimeric vectors, and adenovirus-based vectors, and lentiviral vectors, such as *Herpes simplex* (HSV)-based vectors. These viral vectors can be prepared using standard recombinant DNA techniques described in, for example, Sambrook et al., *Molecular Cloning, a Laboratory Manual*, 2d edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989); and Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Associates and John Wiley & Sons, New York, N.Y. (1994).

A retroviral vector is derived from a retrovirus. Retrovirus is an RNA virus capable of infecting a wide variety of host cells. Upon infection, the retroviral genome integrates into the genome of its host cell and is replicated along with host cell DNA, thereby constantly producing viral RNA and any nucleic acid sequence incorporated into the retroviral genome. As such, long-term expression of a therapeutic factor(s) is achievable when using retrovirus. Retroviruses contemplated for use in gene therapy

are relatively non-pathogenic, although pathogenic retroviruses exist. When employing pathogenic retroviruses, e.g., human immunodeficiency virus (HIV) or human T-cell lymphotropic viruses (HTLV), care must be taken in altering the viral genome to eliminate toxicity to the host. A retroviral vector additionally can be manipulated to render the virus replication-deficient. As such, retroviral vectors are considered particularly useful for stable gene transfer *in vivo*. Lentiviral vectors, such as HIV-based vectors, are exemplary of retroviral vectors used for gene delivery. Unlike other retroviruses, HIV-based vectors are known to incorporate their passenger genes into non-dividing cells and, therefore, can be of use in treating persistent forms of disease.

AAV vectors are viral vectors of particular interest for use in gene therapy protocols. AAV is a DNA virus, which is not known to cause human disease. The AAV genome is comprised of two genes, rep and cap, flanked by inverted terminal repeats (ITRs), which contain recognition signals for DNA replication and packaging of the virus. AAV requires co-infection with a helper virus (i.e., an adenovirus or a *Herpes simplex* virus), or expression of helper genes, for efficient replication. AAV can be propagated in a wide array of host cells including human, simian, and rodent cells, depending on the helper virus employed. An AAV vector used for administration of a nucleic acid sequence typically has approximately 96% of the parental genome deleted, such that only the ITRs remain. This eliminates immunologic or toxic side effects due to expression of viral genes. If desired, the AAV rep protein can be co-administered with the AAV vector to enable integration of the AAV vector into the host cell genome. Host cells comprising an integrated AAV genome show no change in cell growth or morphology (see, e.g., U.S. Patent No. 4,797,368). As such, prolonged expression of therapeutic factors from AAV vectors can be useful in treating persistent and chronic diseases.

Optionally, the isolated or purified nucleic acid molecule, or fragment thereof, upon linkage with another nucleic acid molecule, can encode a fusion protein. The generation of fusion proteins is within the ordinary skill in the art and can involve the use of restriction enzyme or recombinational cloning techniques (see, e.g., GatewayTM (Invitrogen)). See, also, U.S. Patent No. 5,314,995.

In view of the foregoing, the present invention also provides a composition comprising an above-described isolated or purified nucleic acid molecule, optionally in

the form of a vector. The composition can comprise other components as described further herein.

Also in view of the above, the present invention provides a host cell comprising an above-described isolated or purified nucleic acid molecule, optionally in the form of a vector. It is most preferable that the cell of the present invention expresses the vector, such that the oligonucleotide, or fragment thereof, is both transcribed and translated efficiently by the cell. Examples of cells include, but are not limited to, a human cell, a human cell line, *E. coli* (e.g., *E. coli* TB-1, TG-2, DH5 α , XL-Blue MRF' (Stratagene), SA2821 and Y1090), *B. subtilis*, *P. aeruginosa*, *S. cerevisiae*, *N. crassa*, insect cells (e.g., Sf9, Ea4) and others set forth herein below. The host cell can be present in a host, which can be an animal, such as a mammal, in particular a human.

An antibody to human immunodeficiency virus (HIV) envelope glycoprotein that can recognize one or more strains of HIV, wherein the epitope of HIV recognized by the antibody is inducible, and wherein the antibody binding to the epitope is enhanced by the presence of CD4 and the HIV co-receptor, e.g., CXCR4 or CCR5, is also provided by the present invention. Preferably, the antibody is a neutralizing scFv antibody or an Fab fragment. Preferably, the antibody can bind more than one clade of HIV. Preferably, the antibody comprises SEQ ID NO.: 1 or a variant thereof or SEQ ID NO: 2 or a variant thereof. Preferably, the variant retains the ability to bind to the same epitope as that of SEQ ID NO: 1 or 2, respectively, to a greater or lesser extent. While variants can be isolated from naturally occurring sources or be recombinantly produced, such variants also can be synthesized using standard peptide synthesizing techniques well-known to those of ordinary skill in the art (e.g., as summarized in Bodanszky, Principles of Peptide Synthesis, (Springer-Verlag, Heidelberg: 1984)). In particular, the polypeptide can be synthesized using the procedure of solid-phase synthesis (see, e.g., Merrifield, J. Am. Chem. Soc. 85: 2149-54 (1963); Barany et al., Int. J. Peptide Protein Res. 30: 705-739 (1987); and U.S. Patent No. 5,424,398). If desired, this can be done using an automated peptide synthesizer. Removal of the t-butyloxycarbonyl (t-BOC) or 9-fluorenylmethyloxycarbonyl (Fmoc) amino acid blocking groups and separation of the polypeptide from the resin can be accomplished by, for example, acid treatment at reduced temperature. The polypeptide-containing mixture can then be extracted, for instance, with dimethyl ether, to remove non-peptidic

organic compounds, and the synthesized polypeptide can be extracted from the resin powder (e.g., with about 25% w/v acetic acid). Following the synthesis of the polypeptide, further purification (e.g., using high performance liquid chromatography (HPLC)) optionally can be done in order to eliminate any incomplete polypeptides or free amino acids. Amino acid and/or HPLC analysis can be performed on the synthesized polypeptide to validate its identity. For other applications according to the invention, it may be preferable to produce the polypeptide as part of a larger fusion protein, such as by the methods described herein or other genetic means, or as part of a larger conjugate, such as through physical or chemical conjugation, as known to those of ordinary skill in the art and described herein.

The term "antibodies" is used herein in a broad sense and includes both polyclonal and monoclonal antibodies. In addition to intact immunoglobulin molecules, also included in the term "antibodies" are fragments or polymers of those immunoglobulin molecules, and human or humanized versions of immunoglobulin molecules or fragments thereof, as long as they are chosen for their ability to interact with the proteins disclosed herein. The antibodies can be tested for their desired activity using the *in vitro* assays described herein, or by analogous methods, after which their *in vivo* therapeutic and/or prophylactic activities are tested according to known clinical testing methods.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a substantially homogeneous population of antibodies, i.e., the individual antibodies within the population are identical except for possible naturally occurring mutations that may be present in a small subset of the antibody molecules. The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, as long as they exhibit the desired antagonistic activity (See, U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81: 6851-6855 (1984)).

The present inventive monoclonal antibodies can be made using any procedure which produces monoclonal antibodies. For example, monoclonal antibodies of the invention can be prepared using hybridoma methods, such as those described by Kohler and Milstein, *Nature*, 256:495 (1975). In a hybridoma method, a mouse or other
5 appropriate host animal is typically immunized with an immunizing agent to elicit lymphocytes that produce antibodies that will specifically bind to the immunizing agent.

The monoclonal antibodies also can be made by recombinant DNA methods, such as those described in U.S. Pat. No. 4,816,567 (Cabilly et al.). DNA encoding
10 the disclosed monoclonal antibodies can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of antibodies). Libraries of antibodies or active antibody fragments also can be generated and screened using phage display techniques, e.g., as described in U.S. Pat. No. 5,804,440 to
15 Burton et al. and U.S. Patent No. 6,096,551 to Barbas et al.

In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in
20 International Patent Application Publication No. WO 94/29348, published Dec. 22, 1994, and U.S. Pat. No. 4,342,566. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields a fragment that has two antigen combining sites and is still capable of cross-linking antigen.

25 As used herein, the term "antibody or fragments thereof" encompasses chimeric antibodies and hybrid antibodies, with dual or multiple antigen or epitope specificities, single chain antibodies and fragments, such as F(ab')₂, Fab', Fab, scFv and the like, including hybrid fragments. Thus, fragments of the antibodies that retain the ability to bind their specific antigens are provided. For example, fragments of antibodies which
30 maintain HIV gp120 binding activity are included within the meaning of the term "antibody or fragment thereof." Such antibodies and fragments can be made by techniques known in the art and can be screened for specificity and activity according

to the methods set forth in the Examples and in general methods for producing antibodies and screening antibodies for specificity and activity (See Harlow and Lane. Antibodies, A Laboratory Manual. Cold Spring Harbor Publications, New York (1988)). Also included within the meaning of "antibody or fragments thereof" are
5 conjugates of antibody fragments and antigen binding proteins (single chain antibodies) as described, for example, in U.S. Pat. No. 4,704,692, the contents of which are hereby incorporated by reference.

The fragments, whether attached to other sequences or not, can also include insertions, deletions, substitutions, or other selected modifications of particular regions
10 or specific amino acids residues, provided the activity of the antibody or antibody fragment is not significantly altered or impaired compared to the non-modified antibody or antibody fragment. These modifications can provide for some additional property, such as to remove/add amino acids capable of disulfide bonding, to increase bio-longevity, to alter secretory characteristics; etc. In any case, the antibody or
15 antibody fragment must possess a bioactive property, such as specific binding to its cognate antigen. Functional or active regions of the antibody or antibody fragment can be identified by mutagenesis of a specific region of the protein, followed by expression and testing of the expressed polypeptide. Such methods are readily apparent to a skilled practitioner in the art and can include site-specific mutagenesis of the nucleic
20 acid encoding the antibody or antibody fragment (Zoller, M.J. *Curr. Opin. Biotechnol.* 3: 348-354 (1992)).

As used herein, the term "antibody" or "antibodies" can also refer to a human antibody and/or a humanized antibody. Many non-human antibodies (e.g., those derived from mice, rats, or rabbits) are naturally antigenic in humans, and thus can give
25 rise to undesirable immune responses when administered to humans. Therefore, the use of human or humanized antibodies in the methods of the invention serves to lessen the chance that an antibody administered to a human will evoke an undesirable immune response.

Human antibodies also can be prepared using any other technique. Examples of
30 techniques for human monoclonal antibody production include those described by Cole et al. (*Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985)) and by Boerner et al. (*J. Immunol.* 147(1): 86-95 (1991)). Human antibodies (and

fragments thereof) also can be produced using phage display libraries (Hoogenboom et al., *J. Mol. Biol.* 227: 381 (1991); Marks et al., *J. Mol. Biol.* 222: 581 (1991)).

Human antibodies also can be obtained from transgenic animals. For example, transgenic, mutant mice that can produce a full repertoire of human antibodies in
5 response to immunization have been described (see, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA* 90: 2551-255 (1993); Jakobovits et al., *Nature* 362: 255-258 (1993); and Bruggermann et al., *Year in Immunol.* 7: 33 (1993)). Specifically, the homozygous deletion of the antibody heavy chain joining region (*J(H)*) gene in these chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody
10 production, and the successful transfer of the human germ-line antibody gene array into such germ-line mutant mice results in the production of human antibodies upon antigen challenge.

Antibody humanization techniques generally involve the use of recombinant DNA technology to manipulate the DNA sequence encoding one or more polypeptide
15 chains of an antibody molecule. Accordingly, a humanized form of a non-human antibody (or a fragment thereof) is a chimeric antibody or antibody chain (or a fragment thereof, such as an Fv, Fab, Fab', or other antigen-binding portion of an antibody) which contains a portion of an antigen binding site from a non-human (donor) antibody integrated into the framework of a human (recipient) antibody.

20 To generate a humanized antibody, residues from one or more complementarity determining regions (CDRs) of a recipient (human) antibody molecule are replaced by residues from one or more CDRs of a donor (non-human) antibody molecule that is known to have desired antigen binding characteristics (e.g., a certain level of specificity and affinity for the target antigen). In some instances, Fv framework (FR) residues of
25 the human antibody are replaced by corresponding non-human residues. Humanized antibodies may also contain residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. In practice, humanized antibodies are typically human antibodies in which
30 some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies. Humanized antibodies generally contain at least a portion of an antibody constant region (Fc), typically that of a human antibody (Jones

et al., *Nature* 321: 522-525 (1986); Reichmann et al., *Nature* 332: 323-327 (1988); and Presta, *Curr. Opin. Struct. Biol.* 2: 593-596 (1992)).

Methods for humanizing non-human antibodies are well-known in the art. For example, humanized antibodies can be generated according to the methods of Winter
5 and co-workers (Jones et al., *Nature* 321: 522-525 (1986); Riechmann et al., *Nature* 332: 323-327 (1988); and Verhoeyen et al., *Science* 239: 1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Methods that can be used to produce humanized antibodies are also described in U.S. Patent No. 4,816,567 (Cabilly et al.), U.S. Patent No. 5,565,332
10 (Hoogenboom et al.), U.S. Patent No. 5,721,367 (Kay et al.), U.S. Patent No. 5,837,243 (Deo et al.), U.S. Patent No. 5,939,598 (Kucherlapati et al.), U.S. Patent No. 6,130,364 (Jakobovits et al.), and U.S. Patent No. 6,180,377 (Morgan et al.).

A fusion protein or conjugate (conjugate produced by chemical or physical means) comprising an above-described antibody is also provided. The fusion protein or
15 conjugate can comprise an other antibody that binds to an epitope of HIV, such as an antibody to HIV envelope glycoprotein that can recognize one or more strains of HIV, wherein the epitope of HIV recognized by the antibody is inducible, and wherein the antibody binding to the epitope is enhanced by the presence of CD4 and the HIV co-receptor. The other antibody can be a neutralizing scFv antibody fragment or an Fab
20 fragment. Alternatively, the fusion protein or conjugate can comprise CD4, in which case the fusion protein can comprise the amino acid sequence of SEQ ID NO: 7 or a variant thereof, wherein the variant retains the ability to bind to the same epitope as that of SEQ ID NO: 7 to a greater or lesser extent. As another alternative, the fusion protein or conjugate can comprise a toxin.

25 Toxins are poisonous substances produced by plants, animals, or microorganisms that, in sufficient dose, are often lethal. A preferred toxin is Pseudomonas toxin. Diphtheria toxin is a substance produced by *Corynebacterium diphtheria*, which can be used therapeutically. This toxin consists of an α subunit and a β subunit, which, under proper conditions, can be separated. Another example of a
30 toxin is tetanus toxoid, which is produced by *Clostridium tetani*. Lectins are proteins, usually isolated from plant material, which bind to specific sugar moieties. Many lectins are also able to agglutinate cells and stimulate lymphocytes. However, ricin is a

toxic lectin, which has been used immunotherapeutically. This is accomplished by binding the alpha-peptide chain of ricin, which is responsible for toxicity, to the antibody molecule to enable site-specific delivery of the toxic effect. Other therapeutic agents, which can be coupled to the antibodies, are known, or can be easily ascertained, by those of ordinary skill in the art.

Many peptide toxins have a generalized eukaryotic receptor binding domain; in these instances the toxin must be modified to prevent intoxication of cells not bearing the targeted receptor (e.g., to prevent intoxication of cells not bearing the "X" receptor but having a receptor for the unmodified toxin). Any such modifications must be made in a manner which preserves the cytotoxic functions of the molecule. Potentially useful toxins include, but are not limited to: cholera toxin, ricin, Shiga-like toxin (SLT-I, SLT-II, SLT-IIIV), LT toxin, C3 toxin, Shiga toxin, pertussis toxin, tetanus toxin, Pseudomonas exotoxin, alorin, saporin, modeccin, and gelatin. Diphtheria toxin can be used to produce molecules useful as described herein. Diphtheria toxin, whose sequence is known, and hybrid molecules thereof, are described in detail in U.S. Patent No. 4,675,382 to Murphy. The natural diphtheria toxin molecule secreted by *Corynebacterium diphtheriae* consists of several functional domains which can be characterized, starting at the amino terminal end of the molecule, as enzymatically-active Fragment A (amino acids Gly1-Arg193) and Fragment B (amino acids Ser194-Ser535), which includes a translocation domain and a generalized cell binding domain (amino acid residues 475 through 535). The process by which diphtheria toxin intoxicates sensitive eukaryotic cells involves at least the following steps: (i) the binding domain of diphtheria toxin binds to specific receptors on the surface of a sensitive cell; (ii) while bound to its receptor, the toxin molecule is internalized into an endocytic vesicle; (iii) either prior to internalization, or within the endocytic vesicle, the toxin molecule undergoes a proteolytic cleavage between fragments A and B; (iv) as the pH of the endocytic vesicle decreases to below 6, the toxin crosses the endosomal membrane, facilitating the delivery of Fragment A into the cytosol; (v) the catalytic activity of Fragment A (i.e., the nicotinamide adenine dinucleotide--dependent adenosine diphosphate (ADP) ribosylation of the eukaryotic protein synthesis factor termed "Elongation Factor 2") causes the death of the intoxicated cell. A single molecule of Fragment A introduced into the cytosol is sufficient to inhibit the cell's

protein synthesis machinery and kill the cell. The mechanism of cell killing by *Pseudomonas* exotoxin A, and possibly by certain other naturally-occurring toxins, is very similar.

A mixed toxin molecule is a molecule derived from two different polypeptide
5 toxins. Generally, as discussed above in connection with diphtheria toxin, polypeptide toxins have, in addition to the domain responsible for generalized eukaryotic cell binding, an enzymatically active domain and a translocation domain. The binding and translocation domains are required for cell recognition and toxin entry respectively. Naturally-occurring proteins which are known to have a translocation domain include
10 diphtheria toxin, *Pseudomonas* exotoxin A, and possibly other peptide toxins. The translocation domains of diphtheria toxin and *Pseudomonas* exotoxin A are well characterized (see, e.g., Hoch et al., Proc. Natl. Acad. Sci. USA 82:1692, 1985; Colombatti et al., *J. Biol. Chem.* 261: 3030 (1986); and Deleers et al., *FEBS Lett.* 160: 82 (1983)), and the existence and location of such a domain in other molecules may be
15 determined by methods such as those employed by Hwang et al. (Cell 48:129 (1987)); and Gray et al. (PNAS USA 81: 2645 (1984)). A useful mixed toxin hybrid molecule can be formed by fusing the enzymatically active A subunit of *E. coli* Shiga-like toxin (Calderwood et al., PNAS USA 84: 4364 (1987)) to the translocation domain (amino acid residues 202 through 460) of diphtheria toxin, and to a molecule targeting a
20 particular cell type, as described in U.S. Patent No. 5,906,820 to Bacha. The targeting portion of the three-part hybrid causes the molecule to attach specifically to the targeted cells, and the diphtheria toxin translocation portion acts to insert the enzymatically active A subunit of the Shiga-like toxin into the targeted cell. The enzymatically active portion of Shiga-like toxin, like diphtheria toxin, acts on the protein synthesis
25 machinery of the cell to prevent protein synthesis, thus killing the cell.

The targeting molecule (for example, the antibody), and the cytotoxin can be linked in several ways. If the hybrid molecule is produced by expression of a fused gene, a peptide bond serves as the link between the cytotoxin and the antibody or antibody fragment. Alternatively, the toxin and the binding ligand can be produced
30 separately and later coupled by means of a non-peptide covalent bond. For example, the covalent linkage may take the form of a disulfide bond. In this case, the DNA encoding the antibody can be engineered to contain an extra cysteine codon. The

cysteine must be positioned so as to not interfere with the binding activity of the molecule. The toxin molecule must be derivatized with a sulfhydryl group reactive with the cysteine of the modified antibody. In the case of a peptide toxin this can be accomplished by inserting a cysteine codon into the DNA sequence encoding the toxin.

5 Alternatively, a sulfhydryl group, either by itself or as part of a cysteine residue, can be introduced using solid phase polypeptide techniques. For example, the introduction of sulfhydryl groups into peptides is described by Hiskey (*Peptides* 3: 137 (1981)). The introduction of sulfhydryl groups into proteins is described in Maasen et al. (*Eur. J. Biochem.* 134: 32 (1983)). Once the correct sulfhydryl groups are present, the
10 cytotoxin and antibody are purified, both sulfur groups are reduced; cytotoxin and ligand are mixed; (in a ratio of about 1:5 to 1:20) and disulfide bond formation is allowed to proceed to completion (generally 20 to 30 minutes) at room temperature. The mixture is then dialyzed against phosphate buffered saline or chromatographed in a resin such as Sephadex to remove unreacted ligand and toxin molecules.

15 Numerous types of cytotoxic compounds can be joined to proteins through the use of a reactive group on the cytotoxic compound or through the use of a cross-linking agent. A common reactive group that will form a stable covalent bond *in vivo* with an amine is isothiocyanate (Means et al., Chemical Modifications of Proteins, Holden-Day, San Francisco (1971), pp. 105-110). This group preferentially reacts with
20 the ϵ -amine group of lysine. Maleimide is a commonly used reactive group to form a stable *in vivo* covalent bond with the sulfhydryl group on cysteine (Ji, *Methods Enzymol.* 91: 580-609 (1983)). Monoclonal antibodies are incapable of forming covalent bonds with radiometal ions, but they can be attached to the antibody indirectly through the use of chelating agents that are covalently linked to the antibodies.

25 Chelating agents can be attached through amines (Meares et al., *Anal. Biochem.* 142: 68-78 (1984)) and sulfhydryl groups (Koyama, *Chem. Abstr.* 120: 217262t (1994)) of amino acid residues and also through carbohydrate groups (Rodwell et al., *Proc. Natl. Acad. Sci.* 83: 2632-2636 (1986); Quadri et al., *Nucl. Med. Biol.* 20: 559-570 (1993)). Since these chelating agents contain two types of functional groups, one to bind metal
30 ions and the other to joining the chelate to the antibody, they are commonly referred as bifunctional chelating agents (Sundberg et al., *Nature* 250: 587-588 (1974)).

Crosslinking agents have two reactive functional groups and are classified as being homo or heterobifunctional. Examples of homobifunctional crosslinking agents include bismaleimido-hexane (BMH), which is reactive with sulfhydryl groups (Chen et al., *J. Biol. Chem.* 266: 18237-18243 (1991)), and ethylene glycolbis[succinimidylsuccinate] (EGS), which is reactive with amino groups (Browning et al., *J. Immunol.* 143: 1859-1867 (1989)). An example of a heterobifunctional crosslinker is m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) (Myers et al., *J. Immunol. Meth.* 121: 129-142 (1989)). These methodologies are simple and are commonly employed.

10 The above nucleic acid molecules, vectors, host cells, antibodies, fusion proteins and conjugates are preferably administered to a subject as a composition, such as one comprising a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material can be administered to a subject without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier is selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject. Suitable carriers and their formulations are described in *Remington: The Science and Practice of Pharmacy* (19th ed.), A.R. Gennaro, ed., Mack Publishing Company, Easton, PA (1995), and include carriers, thickeners, diluents, buffers, preservatives, surface-active agents, and the like, in addition to the active agent. The pharmaceutical composition also can comprise one or more active ingredients, such as antimicrobial agents, anti-inflammatory agents, anesthetics, and the like. Typically, an appropriate amount of a pharmaceutically acceptable salt is used in the formulation to render the formulation isotonic. Examples of pharmaceutically acceptable carriers include, but are not limited to, saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7 to about 7.5. Further carriers include sustained-release preparations, such as semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that

certain carriers can be preferable depending upon, for instance, the route of administration and concentration of antibody being administered.

The above nucleic acid molecules, vectors, host cells, antibodies, fusion proteins and conjugates can be administered to a human or a collection of cells by injection (e.g., transdermal, intravenous, intraperitoneal, subcutaneous, intramuscular), or by other methods, such as infusion, that ensure delivery to the bloodstream in an effective form. Local or intravenous injection is preferred. Other methods include topical, such as topical intranasal administration or administration by inhalant, vaginal, rectal, ophthalmic, oral, intravenous drop, subcutaneous, and the like.

As used herein, "topical intranasal administration" means delivery of the compositions into the nose and nasal passages through one or both of the nares and can comprise delivery by a spraying mechanism or droplet mechanism, or through aerosolization. Delivery also can be directly to any area of the respiratory system (e.g., lungs) via intubation.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives also can be present such as, for example, antimicrobials, anti-oxidants, chelating agents, inert gases and the like.

Parenteral administration of the composition, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral administration involves the use of a slow-release or sustained-release system such that a constant dosage is maintained. See, e.g., U.S. Patent No. 3,610,795, which is incorporated by reference herein.

The composition (for example, incorporated into microparticles, liposomes, or cells) can be targeted to a particular cell type via antibodies, receptors, or receptor ligands. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Senter et al., *Bioconjugate Chem.* 2: 447-451 (1991);
5 Bagshawe, *Br. J. Cancer* 60: 275-281 (1989); Bagshawe et al., *Br. J. Cancer* 58: 700-703 (1988); Senter et al., *Bioconjugate Chem.* 4:3-9 (1993); Battelli et al., *Cancer Immunol. Immunother.* 35: 421-425 (1992); Pietersz and McKenzie, *Immunolog. Reviews* 129: 57-80 (1992); and Roffler et al., *Biochem. Pharmacol* 42: 2062-2065 (1991)). Vehicles, such as "stealth" and other antibody-conjugated liposomes
10 (including lipid-mediated drug targeting), receptor-mediated targeting of DNA through cell specific ligands, lymphocyte-directed tumor targeting, and highly specific therapeutic retroviral targeting of cells *in vivo*, can be used. The following references are examples of the use of this technology to target specific proteins to tissue (Hughes et al., *Cancer Research* 49: 6214-6220 (1989); and Litzinger and Huang, *Biochimica et*
15 *Biophysica Acta* 1104: 179-187 (1992)). In general, receptors are involved in pathways of endocytosis, either constitutive or ligand induced. These receptors cluster in clathrin-coated pits, enter the cell via clathrin-coated vesicles, pass through an acidified endosome in which the receptors are sorted, and then either recycle to the cell surface, become stored intracellularly, or are degraded in lysosomes. The internalization
20 pathways serve a variety of functions, such as nutrient uptake, removal of activated proteins, clearance of macromolecules, opportunistic entry of viruses and toxins, dissociation and degradation of ligand, and receptor-level regulation. Many receptors follow more than one intracellular pathway, depending on the cell type, receptor concentration, type of ligand, ligand valency, and ligand concentration. Molecular and
25 cellular mechanisms of receptor-mediated endocytosis have been reviewed (Brown and Greene, *DNA and Cell Biology* 10(6): 399-409 (1991)).

Formulations for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like can be necessary or
30 desirable.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders can be desirable.

Some of the compositions potentially can be administered as a pharmaceutically acceptable acid- or base-addition salt, formed by reaction with inorganic acids, such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base, such as sodium hydroxide, ammonium hydroxide, and potassium hydroxide, and organic bases, such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

Effective dosages and schedules for administering the above nucleic acid molecules, vectors, host cells, antibodies, and fusion proteins can be determined empirically, and making such determinations is within the skill in the art. Those skilled in the art will understand that the dosage that must be administered will vary depending on, for example, the subject, the route of administration, whether a nucleic acid molecule, vector, host cell, antibody, fusion protein or conjugate is being administered, and whether other drugs being administered, not to mention the age, condition, and gender of the human and the extent of disease. Guidance in selecting appropriate doses for antibodies (or fusion proteins comprising same) is found in the literature on therapeutic uses of antibodies, e.g., Handbook of Monoclonal Antibodies, Ferrone et al., eds., Noyes Publications, Park Ridge, N.J. (1985), Ch. 22 and pp. 303-357; Smith et al., Antibodies in Human Diagnosis and Therapy, Haber et al., eds., Raven Press, New York (1977), pp. 365-389. A typical daily dosage of the antibody used alone can range from about 1 μ g/kg up to about 100 mg/kg of body weight or more per day, depending on the factors mentioned above. For example, the range can be from about 100 mg to one gram per dose. Nucleic acids, vectors and host cells should be administered so as to result in comparable levels of production of antibodies or fusion proteins thereof.

Following administration of a nucleic acid molecule, vector, host cell, antibody, fusion protein or conjugate for treating, inhibiting, or reducing the severity of an HIV infection, the efficacy of the therapeutic agent can be assessed in various ways well-known to the skilled practitioner. For instance, one of ordinary skill in the art will

understand that an antibody of the invention is efficacious in treating or inhibiting an HIV infection in a subject by observing that the antibody reduces viral load or prevents a further increase in viral load. Viral loads can be measured by methods that are known in the art, for example, using polymerase chain reaction assays to detect the presence of HIV nucleic acid or antibody assays to detect the presence of HIV protein in a sample (e.g., but not limited to, blood) from a subject or patient, or by measuring the level of circulating anti-HIV antibody levels in the patient. Efficacy of the antibody treatment also can be determined by measuring the number of CD4⁺ T cells in the HIV-infected subject. An antibody treatment that inhibits an initial or further decrease in CD4⁺ T cells in an HIV-positive subject or patient, or that results in an increase in the number of CD4⁺ T cells in the HIV-positive subject, is an efficacious antibody treatment.

The nucleic acid molecules, vectors, host cells, antibodies, fusion proteins and/or conjugates of the invention can be administered prophylactically to patients or subjects who are at risk for being exposed to HIV or who have been newly exposed to HIV. In subjects who have been newly exposed to HIV but who have not yet displayed the presence of the virus (as measured by PCR or other assays for detecting the virus) in blood or other body fluid, efficacious treatment with an antibody of the invention partially or completely inhibits the appearance of the virus in the blood or other body fluid.

The nucleic acid molecules, vectors, host cells, antibodies, fusion proteins and/or conjugates of the invention can be combined with other well-known therapies and prophylactic vaccines already in use. Such combinations can generate an additive or a synergistic effect with current treatments. The nucleic acid molecules, vectors, host cells, antibodies and/or conjugates of the invention can be combined with HIV and AIDS therapies and vaccines such as highly active antiretroviral therapy (HAART), AZT, structured treatment interruptions of HAART, cytokine immune enhancement therapy (IL-2, IL-12, CD40L+ IL-12, IL-7, IFNs), cell replacement therapy, recombinant viral vector vaccines, DNA vaccines, inactivated virus preparations, and immunosuppressive agents, such as Cyclosporin A. Such therapies can be administered in the manner already in use for the known treatment providing a therapeutic or prophylactic effect (Silvestri and Feinberg "Immune Intervention in AIDS." In

Immunology of Infectious Disease. H.E. Kauffman, A. Sher, and R. Ahmed eds., ASM Press. Washington DC. (2002)).

As described above, the compositions can be administered in a pharmaceutically acceptable carrier and can be delivered to the subject's cells *in vivo* and/or *ex vivo* by a variety of mechanisms well-known in the art (e.g., uptake of naked DNA, liposome fusion, intramuscular injection of DNA via a gene gun, endocytosis and the like).

If *ex vivo* methods are employed, cells or tissues can be removed and maintained outside the body according to standard protocols well-known in the art. Compositions comprising a nucleic acid, optionally in the form of a vector encoding the antibody or fusion protein comprising same, can be introduced into the cells via any gene transfer mechanism, such as, for example, calcium phosphate mediated gene delivery, electroporation, microinjection or proteoliposomes. The transduced cells then can be infused (e.g., in a pharmaceutically acceptable carrier) or homotopically transplanted back into the subject per standard methods for the cell or tissue type. Standard methods are known for transplantation or infusion of various cells into a subject.

Thus, in view of the above, the present invention provides a method of inhibiting an infection of a human at risk of becoming infected with HIV. The method comprises administering to the human an infection-inhibiting amount of an above-described nucleic acid, vector, host cell, antibody or fusion protein, whereupon the infection of the human is inhibited. The HIV can be HIV-1 or HIV-2. One of ordinary skill in the art will appreciate that, in the context of HIV infection and AIDS, any degree of inhibition of infection can be beneficial. Preferably, the infection is inhibited to such a degree that the human does not evidence the signs and symptoms of infection and preferably does not develop AIDS.

Also in view of the above, the present invention provides a method of reducing the severity of an infection of a human infected with HIV, such as HIV-1 or HIV-2. The method comprises administering to the human a severity of infection-reducing amount of an above-described nucleic acid, vector, host cell, antibody or fusion protein, whereupon the severity of the infection of the human is reduced. One of ordinary skill in the art will appreciate that, in the context of HIV infection and AIDS, any degree of

reduction in the severity of infection can be beneficial. Preferably, the reduction in the severity of infection is to such a degree that the human does not evidence the signs and symptoms of infection and preferably does not develop AIDS or, in the event that the human already has been diagnosed with AIDS, preferably the human does not
5 experience an increase in the severity of AIDS.

Still also in view of the above, the present invention provides a method of treating an infection, such as a chronic infection, of a human with HIV, such as HIV-1 or HIV-2. The method comprises administering to the human an infection-treating amount of an above-described nucleic acid, vector, host cell, antibody or fusion protein,
10 whereupon the infection of the human is treated. One of ordinary skill in the art will appreciate that, in the context of HIV infection and AIDS, any inhibition or amelioration of infection is beneficial. Preferably, the infection is treated to such a degree that the human does not evidence a worsening of the signs and symptoms of infection and preferably does not develop AIDS or, in the event that the human already
15 has been diagnosed with AIDS, preferably the human does not experience an increase in the severity of AIDS. Also preferably, if the human is infected but has not yet developed AIDS, the human's condition improves such that he/she becomes essentially asymptomatic and does not evidence signs of infection.

20

EXAMPLE

The following example serves to illustrate the present invention and is not intended to limit its scope in any way.

Selection of two phage scFvs (m6 and m9) with high affinity for different Env-
25 CD4 complexes by SAP.

We hypothesized that, by sequential antigens during panning of phage display libraries and by screening the enriched libraries using different antigens, the selected phage will display scFvs against conserved epitopes shared among all antigens used during the entire selection process. Complexes of two different recombinant soluble
30 Envs (gp140_{89.6} and gp140_{IIIb}) with two-domain soluble CD4 (sCD4) were used as antigens for phage library panning as described in the Experimental Protocol. Screening of individual phage clones after panning was performed in phage ELISA

with gp140_{89.6}, gp120_{JR-FL}, gp140_{IIIB}, and their complexes with sCD4. Two phage clones, designated m6 and m9, bound significantly to all antigens and were selected for further characterization. Phagemid DNA of m6 and m9 was prepared and sequenced. The two clones exhibited differences in the amino acid composition.

5 Binding of soluble Fab m6 and Fab m9 to gp120 and gp140 from different isolates is significantly increased by CD4.

m6 bound gp120 from the primary isolate 89.6 with a high affinity (nM), which was significantly increased after binding of sCD4 to gp120 as measured by an ELISA assay. Interestingly, although the affinity of m6 to gp120 alone was comparable to that
10 of X5, the affinity to the 120-sCD4 was significantly increased.

Inhibition of Env-mediated membrane fusion.

To determine the breadth and potency of HIV neutralization by m6 we measured the ability to inhibit cell-cell fusion mediated by Envs of primary isolates
15 from different clades in comparison with the potent broadly HIV neutralizing Fab X5 (Table 1). m6 inhibited cell-cell fusion mediated by Envs of primary isolates from different clades with a potency comparable (3 isolates), worse (2 isolates) or better (6 isolates) than X5 as measured by a cell fusion assay. These results suggest that m6 is a potent broadly HIV-1 neutralizing scFv antibody.

20

Experimental protocol.

Construction of the X5 scFv library.

The X5 scFv library was generated using standard approaches. Briefly, the X5 variable regions of heavy chain (VH) and light chain (VL) were amplified by PCR
25 using two pairs of primer: VL5Sac and VL3Link for VL; VH5Link and VH3Spe for VH. X5 scFv resulted from SOE-PCR of VL and VH. PCR products were gel-purified, digested with *Sac* I and *Spe* I and gel-purified again. Purified fragment was then cloned to phagemid pComb3X linearized by *Sac* I and *Spe* I. To introduce point mutations to X5 scFv construct, we did DNA random mutagenesis with the GeneMorph
30 PCR Mutagenesis Kit (Stratagene, La Jolla, CA) according to manufacture's instruction for high range mutation frequency with slight modification. The 1st PCR reaction was carried out in a total volume of 50 µl by adding 10 pg of scFv DNA (60 pg of the

recombinant phagemid DNA), 20 pmol of primer VL5Sac and VH3Spe and 2.5 units of mutazyme under the following conditions: an initial denaturation for 5 min at 94°C followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a filling cycle of 72°C for 10 min. The 1st PCR products were gel-purified and 25 ng of purified 1st PCR products were used as templates in the 2nd PCR amplification under the same conditions as described above. The products from the 2nd PCR amplification were gel-purified and digested with *Sac* I and *Spe* I. The pComb3X containing the Fab X5 insert was digested with the same enzymes, and the resulting linearized vector was purified by agarose gel electrophoresis. The vector backbone was excised from the gel. The purified vector DNA was ligated with purified scFv fragments, and the ligation products were electroporated into electrocompetent *E. coli* XL1-blue cells to create an scFv mutagenesis library. Four separated ligations and transformations were pooled to increase the library diversity. The efficiency of this transformation with a total of 320 ng of purified scFv fragments yielded 1.2×10^6 independent transformants. Forty individual clones were randomly selected, and plasmid DNA was prepared and sequenced. The average mutation rate of 40 clones was 6 bases per kb DNA.

An scFv phage library was prepared from initial transformations upon infection with the replication-defective helper phage M13KO7. The phage titer was determined by the addition of dilutions to exponentially growing *E. coli* XL1-blue cells.

20

Sequential antigen panning of the scFv mutagenesis library.

Phage (5×10^{12} cfu/ml) were preadsorbed on streptavidin-M280-Dynabeads in PBS for 1 h at room temperature (RT) followed by depletion in an immunotube (Nunc, Denmark) coated with 10 µg/ml sCD4 for 1 h at 37°C. A depleted phage library was incubated with 50 nM biotinylated gp140_{89.6} complexed with sCD4 in solution (molar ratio of gp140_{89.6} to sCD4 = 1:1) for 2 h at RT with gentle agitation. Phage binding to biotinylated Env were separated from the phage library using streptavidin-M280-Dynabeads and a magnetic separator (Dyna). The beads were washed 20 times with 1 ml of PBS containing 0.1% Tween-20 and another 20 times with 1 ml of PBS. Bound phage were eluted by incubation at RT for 10 min with 1 ml of 100 mM TEA, followed by neutralization with 0.5 ml of 1 M, pH 7.5, Tris-HCl. Eluted phage were rescued by infection of *E. coli* TG1 cells, and a phage library was prepared for the next round of

panning. For the 2nd round of panning, 10 nM (2 nM for the 3rd round) of biotinylated gp140_{89,6} complexed with sCD4 (1:1 on molar level) was used as antigen. For the 4th round of panning, 2 nM of biotinylated gp140_{IIIb} complexed with sCD4 (1:1 on molar level) was used as antigen. After the 3rd and 4th rounds of panning, 20 individual clones
5 were screened by phage ELISA for binding to gp140_{89,6}, gp120_{JRFL}, gp140_{IIIb} and their complexes with sCD4 as follows. Single colonies were inoculated into 1 ml of 2 x YT medium containing 100 µg/ml ampicillin and 2% glucose in 12-ml falcon tubes. The tubes were incubated overnight at 37 °C / 250 rpm. Ten µl of overnight culture from each tube were inoculated into 1 ml of 2 x YT medium containing 100 µg/ml
10 ampicillin, 2% glucose and about 4 x 10⁹ cfu/ml of M₁₃KO₇ in 12-ml falcon tubes. The phage tubes were incubated at 37 °C / 250 rpm for 2 h and centrifuged at 4,000 rpm for 10 min at RT. The supernatant was removed and the cells were suspended in 1 ml of 2x YT medium with 100 µg/ml ampicillin and 50 µg/ml kanamycin. The tubes were then incubated overnight at 30 °C / 250 rpm. After 16 h, the tubes were centrifuged at
15 4,000 rpm for 10 min at 4°C. The supernatant was used for phage ELISA.

Phage ELISA.

Phage ELISA was performed by using 96-well Nunc-ImmunoTM MaxisorpTM surface plates (Nalge Nunc International, Denmark), which were coated overnight at
20 4°C with 100 µl of gp120/140 (1 µg/ml in sodium bicarbonate buffer, pH 8.3) or gp120/140-sCD4 complex (100 µg/ml gp120/140 in PBS were premixed with an equal volume of 100 µg/ml sCD4). After incubation at RT for 30 min, the mixture was diluted to 1 µg/ml in PBS, and blocked in 100 µl of 4 % non-fat dry milk in PBS for 1 hour at 37°C. After 4 washes with 0.05% Tween20/PBS washing buffer (WB), wells
25 were incubated with 100 µl of phage supernatant for 2 hour at 37°C. Bound phage were detected by using horseradish peroxidase (HRP) conjugated anti-M13 monoclonal antibody (Pharmacia) with incubation for 1 h at 37°C and revealed by adding ready-to-use ABTs substrate (Pharmacia). Color development was performed at RT for 15 min and monitored at 405 nm.

Preparation of soluble scFv fragments.

The pComb3X phagemids containing m6 and m9 scFv genes were prepared and transformed to *E. coli* Top 10. His 6-tagged soluble scFvs were expressed and purified
5 by IMAC using Ni-NTA resin according to manufacturer's protocols.

Enzyme-linked immunosorbent assays (ELISAs).

ELISA was performed by using 96-well Nunc-Immuno™ Maxisorp™ surface plates. Coating of antigen and washing and blocking steps were the same as
10 described for phage ELISA. For scFv binding assay, microplate wells were incubated with 100 µl two-fold serially diluted biotinylated soluble scFv for 2 hours at 37°C. After 4 washes with WB, 100 µl of a 1:2,500 dilution of HRP-streptavidin were added and incubated for 1 hour at 37°C. Following 4 washes with WB, the assay was developed at 37°C for 15-30 minutes with ready-to-use ABTs substrate and monitored
15 at 405 nm. For competition ELISA, 50 µl of two-fold serially diluted competing scFv hmAbs (m6, m9 or scFv X5) were added to the blocked and washed wells, immediately followed by addition of 50 µl of Fab or IgG hmAbs (X5, IgG 17b, IgG b12) previously determined to result in an ELISA signal that was 50 to 75% of maximum without competitor. After incubation for 2 h at 37°C, the wells were washed as above, probed
20 with a HRP-conjugated anti-human IgG F(ab')₂ conjugate (Pierce) diluted 1:2,500 in PBS containing 2% non-fat dry milk and detected as above.

Cell-cell fusion assay.

Cells (10⁵ 293 cells) transfected with plasmids encoding various Envs under the
25 control of the T7 promoter and infected with recombinant vaccinia virus encoding the T7 polymerase gene, were preincubated with m6 or X5 at 50 µg/ml for 30 min at 37°C, and then mixed with 10⁵ CEM-CCR5 cells. The extent of cell fusion was quantified by counting the number of syncytia 12 h later. The data are shown on Table 1, in which the data are averages of duplicate samples and presented as % of fusion inhibition.

Table 1: Inhibition of cell fusion mediated by Envs from various clades by m6 and X5.

Env	UG0	RW0	US7	HT5	US0	89.6	NL	BR0	TH0	BR0	UG9
	37.8	20.5	15.6	93.1	05.11		4-3	25.9	22.4	19.4	75.10
Clade	A	A	B	B	B	B	B	C	EA	F/B	G
m6	66	56	27	50	83	98	100	57	72	37	48
X5	34	45	56	27	72	93	83	71	43	37	19

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Infectious virus neutralization assay.

This HIV-1 neutralization assay is based on the use of infectious virus and a reporter-gene cell line BC-53. Primary HIV-1 isolates were either isolated from Institutional Review Board-approved CDC Studies or obtained from the NIH Research and Reference Reagent Program through the WHO collaborative network. The detailed characteristics of various isolates, including subtype determination based on the envelope region and coreceptor usage using GHOST cell lines has been previously described (15-17). Viral stocks were generated by infection of CD8 depleted normal human peripheral blood mononuclear cells as previously described (15, 16). Viral stocks were filtered through 0.22 μ m filters, aliquoted and maintained at -70°C prior to use. A reporter gene based viral replication assay was used to read out the viral replication (18). Briefly, JC53-BL, an HIV-1 indicator cell line derived from HeLa cells that express high levels of CD4, CXCR4 and CCR5, contains reporter cassettes for luciferase and β -galactosidase both driven by the HIV-1 LTR (a kind gift of Tranzyme Inc. Birmingham Al). These cassettes allow detection of HIV-1 infection (tat production) by measuring either luciferase activity with a luminometer or by counting blue foci after staining the cells with X-gal. The JC53-BL cells are maintained in c-DMEM, which is DMEM supplemented with 10% fetal calf serum (Hyclone), 2mM glutamine (Gibco), 100 units per ml penicillin G, and 100ug/ml

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streptomycin (Gibco). The viral titers are determined by adding serial dilutions of the virus stocks in C-DMEM media containing 40 µg/ml DEAE-dextran to 20,000 JC53-BL cells per well in duplicate in 96 well plates. Following a 48 hour incubation at 37°C in a 5% CO₂ incubator, the cells are fixed and stained. Blue foci are counted using a standard light microscope, and the titers are expressed as infectious units or blue foci units per ml. From the infectious unit data, MOI values are determined for the inhibition assays.

The neutralizing activities of the antibodies in this assay were determined as follows. The JC53-BL cells were removed from T-150 flasks using 0.017M PBS, 0.1mM EDTA at a pH of 7.4 approximately 18 hours prior to starting the inhibition assays and were plated at a density of 20,000 cells per well in white 96 well plates in 50 µl of C-DMEM. Viral stocks (MOI range of 0.009 to 0.65) were pre-incubated with different concentrations of the mAbs (final concentrations of 100-0.05µg/ml) for 1 hour, prior to addition to the cells in media containing 40 µg/ml DEAE-dextran to give a final volume of 200 µl per well. The plates were incubated in a 37°C, 5% CO₂ incubator for 48 hour incubation, and luciferase activity was measured using the Steady-Glo Luciferase Assay System (Promega, Madison, WI) following the manufacturer's lysis protocol. The light intensity was measured using a Tecan luminometer with Magellan software (Tecan, Research Triangle Park, NC), and values were calculated as relative light units (RLU). Percent inhibition was calculated by the following formula $1 - ((\text{average RLU of mAb containing wells} / \text{average RLU of virus only wells}) \times 100 = \% \text{ inhibition}$. All assays were performed in duplicates and some in triplicates. The data are presented in Table 2.

Table 2: Neutralization of diverse HIV-1 isolates by m6, m9 and scFv X5.

Virus ^a	Subtype gag/env	Coreceptor	Inhibition (%)		
			m6	m9	scFv X5
			50ug/ml	50ug/ml	50ug/ml
92US714	/B	R5	94	94	60
92US727	/B	R5	22	24	73
92BR023	C/B	R5	82	100	57
92HT593	/B	R5X4	85	88	63
93US151	/B	R5X4	100	100	69
92US076	/B	R5X4	47	52	28
92UG031	A/A	R5	76	79	29
92UG037	A/A	R5	13	44	65
97USSN54	A/A	R5	11	11	6
92RW024	D/A	R5	45	74	42
92RW009	C/A	R5X4	81	95	57
97ZA003	C/C	R5	67	72	25
98CN006	C/C	R5	92	96	34
98IN017	/C	X4	91	93	30
92TH001	A/E	R5	18	31	58
93TH073	A/E	R5	46	31	23
93TH060	A/E	R5	58	40	48
HM16	A/E	X4	100	96	nd
HM14	A/E	X4	81	45	52
CMU 08	A/E	R5X4	98	100	35
93BR019	/BF	R5	50	29	66
93BR029	B/F	R5	58	45	30
93BR020	F/F	R5X4	39	19	25
JV1083	/G	R5	42	29	22
HIV-G3	/G	R5	53	63	14
YBF-30	Grp N	R5	71	85	14
BCF-01	Grp O	R5	10	0	0
BCF-02	Grp O	R5	32	7	46
BCF-03	Grp O	R5	0	0	3
5084/5-83§	B	R5	70	85	53
5084/10-86AIDS§	B	R5X4	71	87	18
5048/7-82§	B	R5	51	83	0
5048/3-91AIDS§	B	R5X4	96	100	45

^a: HIV-1 isolates. Their subtypes were determination based on the envelope
 5 region and coreceptor usage using GHOST cell lines (1-5). nd – not determined
 §: virus isolated from rapid progressor (5084) or late progressor (5048) with dates
 of isolations and disease status stated. Both patients had switch in their coreceptor
 usage over time.

Inhibition of pseudovirus entry assay.

In this assay format single-round infectious molecular clones, produced by envelope complementation, were used. The degree of virus neutralization by antibody was achieved by measuring luciferase activity. Briefly, 2×10^4

5 U87.CD4.CCR5.CXCR4 cells in 100 μ l of medium (DMEM containing 15% FBS, 1 μ g of puromycin/ml, 300 μ g of G418/ml, glutamine, and penicillin-streptomycin) were added to microplate wells (96-well flat-bottom; Corning Inc., Corning, N.Y.) and incubated for 24 h at 37°C in 5% CO₂. One hundred microliters of medium containing

10 an amount of virus previously determined to yield ~100,000 counts was mixed with various amounts of antibody, incubated for 1 h at 37°C, added to the cells, and incubated for a further 3 days. The wells were aspirated and washed once with PBS, and 60 μ l of luciferase cell culture lysis reagent (Promega, Madison, Wis.) were added. The wells were scraped and the lysate was mixed by pipetting, 50 μ l were transferred to a round-bottom plate (Corning), and the plate was centrifuged at $1,800 \times g$ for 10 min at

15 4°C. Twenty microliters were transferred to an opaque assay plate (Corning), and the luciferase activity was measured on a luminometer (EG&G Berthold LB 96V; Perkin Elmer, Gaithersburg, Md.) by using luciferase assay reagent (Promega). The data are presented in Table 3.

20 Table 3: Neutralizing activity of m6, m9 and scFv X5 for selected HIV-1 isolates measured by an assay based on pseudovirus.

Virus	m6		m9		scFv X5	
	IC50	IC90	IC50	IC90	IC50	IC90
HxB2	1	6	1	5	1	7
JRC5F	5	30	3	15	5	30
YU2	3	100	2	25	50	>100
89.6	2	26	0.1	5	4	8

PBMC-based assay for neutralization of infectious HIV.

The PBMC-based neutralization assay was performed as follows. Serial two-

25 fold dilutions of Abs in 50 μ l were incubated with an equal volume of virus containing 100 TCID₅₀ for 1 h at 37°C and added to 100 μ l of PHA-activated PBMC (5×10^5 /ml). The calculated neutralization titers refer to the Ab concentration present during this

incubation step. After overnight incubation, the cells were washed twice with tissue culture medium. On day 4, 100 µl of medium were replaced with fresh tissue culture medium. Triplicate samples were taken on days 4 and 7, treated with 1% Empigen (Calbiochem) and tested for p24 Ag content using an in-house ELISA. When the values for the p24 concentration at the day 7 were saturated, data for day 4 were used for analysis. The data are presented in Table 4.

Table 4: Standard p24 PBMC assay

Virus	IC90s in µg/ml				
	IgG X5	Fab X5	scFv X5	m6	m9
89.6	>300	>100	12	18	6
93BR020	>300	>100	>50	>50	25
Jrcsf	>300	100	12	8	4

The data are the average of two experiments done in triplicate.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains. The references disclosed are also individually and specifically incorporated by reference herein for the material contained in them that is discussed in the sentence in which the reference is relied upon. If the number designation is not associated with a particular number it will be clear to one of skill which reference is being referred to by the context the reference is relied upon and by a review of the various possible references. The examples all refer to a particular set of references, and thus do not have a letter designation associated with individual numbers.

It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. It will also be apparent to those skilled in the art that nucleic acid sequences are disclosed by the disclosure of the amino acid sequences as one skilled in the art will know what nucleic acids comprise an amino acid. Other embodiments of the invention will be apparent to those skilled in the art from

consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

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What is claimed is:

1. An isolated or purified nucleic acid molecule comprising the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 1 or a variant thereof,
5 wherein the variant retains the ability to bind to the same epitope as that of SEQ ID NO: 1 to a greater or lesser extent.
2. A vector comprising the isolated or purified nucleic acid molecule of claim 1.
10
3. A composition comprising the isolated or purified nucleic acid molecule of claim 1, optionally in the form of a vector.
4. A host cell comprising the isolated or purified nucleic acid molecule of
15 claim 1, optionally in the form of a vector.
5. An isolated or purified nucleic acid molecule comprising the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 2 or a variant thereof, wherein the variant retains the ability to bind to the same epitope as that of SEQ ID
20 NO: 2 to a greater or lesser extent.
6. A vector comprising the isolated or purified nucleic acid molecule of claim 5.
- 25 7. A composition comprising the isolated or purified nucleic acid molecule of claim 5, optionally in the form of a vector.
8. A host cell comprising the isolated or purified nucleic acid molecule of claim 5, optionally in the form of a vector.
30
9. An isolated or purified nucleic acid molecule comprising the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 8 or a variant thereof,

wherein the variant retains the ability to bind to the same epitope as that of SEQ ID NO: 8 to a greater or lesser extent.

10. A vector comprising the isolated or purified nucleic acid molecule of
5 claim 9.

11. A composition comprising the isolated or purified nucleic acid molecule of claim 9, optionally in the form of a vector.

12. A host cell comprising the isolated or purified nucleic acid molecule of
10 claim 9, optionally in the form of a vector.

13. An antibody to human immunodeficiency virus (HIV) envelope glycoprotein that can recognize one or more strains of HIV, wherein the epitope of HIV
15 recognized by the antibody is inducible, and wherein the antibody binding to the epitope is enhanced by the presence of CD4 and the HIV co-receptor.

14. The antibody of claim 13, wherein the antibody is a neutralizing scFv antibody fragment or an Fab fragment.
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15. An antibody of claim 13 or 14, wherein the co-receptor is CXCR4.

16. An antibody of claim 13 or 14, wherein the co-receptor is CCR5.

17. An antibody of claim 13 or 14, wherein the antibody can bind more than
25 one clade of HIV.

18. An antibody of claim 13, wherein the antibody comprises SEQ ID NO.: 1 or a variant thereof, wherein the variant retains the ability to bind to the same epitope
30 as that of SEQ ID NO: 1 to a greater or lesser extent.

19. An antibody of claim 13, wherein the antibody comprises SEQ ID NO.: 2 or a variant thereof, wherein the variant retains the ability to bind to the same epitope as that of SEQ ID NO: 2 to a greater or lesser extent.

5 20. A fusion protein or conjugate comprising the antibody of claim 13, 14, 18 of 19.

 21. The fusion protein or conjugate of claim 20 comprising an other antibody that binds to an epitope of HIV.

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 22. The fusion protein or conjugate of claim 21, wherein the other antibody that binds to an epitope of HIV is an antibody to HIV envelope glycoprotein that can recognize one or more strains of HIV, wherein the epitope of HIV recognized by the antibody is inducible, and wherein the antibody binding to the epitope is enhanced by
15 the presence of CD4 and the HIV co-receptor.

 23. The fusion protein or conjugate of claim 22, wherein the other antibody is a neutralizing scFv antibody fragment or an Fab fragment.

20 24. The fusion protein or conjugate of claim 20, wherein the fusion protein comprises CD4.

 25. The fusion protein or conjugate of claim 24, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 8 or a variant thereof, wherein the
25 variant retains the ability to bind to the same epitope as that of SEQ ID NO: 8 to a greater or lesser extent.

 26. The fusion protein or conjugate of claim 20, wherein the fusion protein comprises a toxin.

30

 27. The fusion protein or conjugate of claim 26, wherein the toxin is Pseudomonas toxin.

28. A composition comprising the antibody of claim 13, 14, 18 or 19, optionally as part of a fusion protein or conjugate.

5 29. A method of inhibiting an infection of a human at risk of becoming infected with HIV, which method comprises administering to the human an isolated or purified nucleic acid molecule encoding an antibody to HIV envelope glycoprotein that can recognize one or more strains of HIV, wherein the epitope of HIV recognized by the antibody is inducible, wherein the antibody binding to the epitope is enhanced by
10 the presence of CD4 and the HIV co-receptor, wherein the antibody is optionally part of a fusion protein, and wherein the isolated or purified nucleic acid molecule is optionally in the form of a vector and/or optionally contained within a cell, whereupon the nucleic acid molecule expresses an infection-inhibiting amount of the antibody in the human and the infection of the human is inhibited.

15 30. The method of claim 29, wherein the antibody is a neutralizing scFv antibody fragment or an Fab fragment.

20 31. The method of claim 29, wherein the isolated or purified nucleic acid molecule encodes the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, or a variant of either of the foregoing, wherein the variant retains the ability to bind to the same epitope as that of SEQ ID NO: 1 or SEQ ID NO: 2, respectively, to a greater or lesser extent, any of which is optionally part of a fusion protein.

25 32. The method of any of claims 29-31, wherein the fusion protein comprises an other antibody that binds to an epitope of HIV.

30 33. The method of claim 32, wherein the other antibody that binds to an epitope of HIV is an antibody fragment to HIV envelope glycoprotein that can recognize one or more strains of HIV, wherein the epitope of HIV recognized by the antibody is inducible, and wherein the antibody binding to the epitope is enhanced by the presence of CD4 and the HIV co-receptor.

34. The method of any of claims 29-31, wherein the fusion protein comprises CD4.

5 35. The method of claim 34, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 8 or a variant thereof, wherein the variant retains the ability to bind to the same epitope as that of SEQ ID NO: 8 to a greater or lesser extent.

10 36. The method of any of claims 29-31, wherein the fusion protein comprises a toxin.

37. The method of claim 36, wherein the toxin is Pseudomonas toxin.

15 38. The method of claim 29, wherein the cell is autologous.

39. The method of claim 38, wherein the cell is a B lymphocyte.

20 40. The method of claim 29, wherein the HIV is HIV type 1 (HIV-1) or HIV type 2 (HIV-2).

25 41. A method of inhibiting an infection of a human at risk of becoming infected with HIV, which method comprises administering to the human an infection-inhibiting amount of an antibody to HIV envelope glycoprotein that can recognize one or more strains of HIV, wherein the epitope of HIV recognized by the antibody is inducible, wherein the antibody binding to the epitope is enhanced by the presence of CD4 and the HIV co-receptor, and wherein the antibody is optionally part of a fusion protein or conjugate, whereupon the infection of the human is inhibited.

30 42. The method of claim 41, wherein the antibody is a neutralizing scFv antibody fragment or an Fab fragment.

43. The method of claim 41, wherein the antibody comprises the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, or a variant of either of the foregoing, wherein the variant retains the ability to bind to the same epitope as that of SEQ ID NO: 1 or SEQ ID NO: 2, respectively, to a greater or lesser extent.

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44. The method of any of claims 41-43, wherein the fusion protein comprises an other antibody that binds to an epitope of HIV.

45. The method of claim 44, wherein the other antibody that binds to an epitope of HIV is an antibody to HIV envelope glycoprotein that can recognize one or more strains of HIV, wherein the epitope of HIV recognized by the antibody is inducible, and wherein the antibody binding to the epitope is enhanced by the presence of CD4 and the HIV co-receptor.

46. The method of any of claims 41-43, wherein the fusion protein comprises CD4.

47. The method of claim 46, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 8 or a variant thereof, wherein the variant retains the ability to bind to the same epitope as that of SEQ ID NO: 8 to a greater or lesser extent.

48. The method of any of claims 41-43, wherein the fusion protein comprises a toxin.

49. The method of claim 48, wherein the toxin is Pseudomonas toxin.

50. The method of claim 41, wherein the HIV is HIV-1 or HIV-2.

51. A method of reducing the severity of an infection of a human infected with HIV, which method comprises administering to the human an isolated or purified nucleic acid molecule encoding an antibody to HIV envelope glycoprotein that can recognize one or more strains of HIV, wherein the epitope of HIV recognized by the

antibody is inducible, wherein the antibody binding to the epitope is enhanced by the presence of CD4 and the HIV co-receptor, wherein the antibody is optionally part of a fusion protein, and wherein the isolated or purified nucleic acid molecule is optionally in the form of a vector and/or optionally contained within a cell, whereupon the nucleic acid molecule expresses a severity of infection-reducing amount of the antibody in the human and the severity of the infection of the human is reduced.

52. The method of claim 51, wherein the antibody is a neutralizing scFv antibody fragment or an Fab fragment.

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53. The method of claim 51, wherein the isolated or purified nucleic acid molecule encodes the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, or a variant of either of the foregoing, wherein the variant retains the ability to bind to the same epitope as that of SEQ ID NO: 1 or SEQ ID NO: 2, respectively, to a greater or lesser extent, any of which is optionally part of a fusion protein.

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54. The method of any of claims 51-53, wherein the fusion protein comprises an other antibody that binds to an epitope of HIV.

55. The method of claim 54, wherein the other antibody that binds to an epitope of HIV is an antibody to HIV envelope glycoprotein that can recognize one or more strains of HIV, wherein the epitope of HIV recognized by the antibody is inducible, and wherein the antibody binding to the epitope is enhanced by the presence of CD4 and the HIV co-receptor.

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56. The method of any of claims 51-53, wherein the fusion protein comprises CD4.

57. The method of claim 56, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 8 or a variant thereof, wherein the variant retains the ability to bind to the same epitope as that of SEQ ID NO: 8 to a greater or lesser extent.

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58. The method of any of claims 51-53, wherein the fusion protein comprises a toxin.

5 59. The method of claim 58, wherein the toxin is *Pseudomonas* toxin.

60. The method of claim 51, wherein the cell is autologous.

61. The method of claim 60, wherein the cell is a B lymphocyte.

10

62. The method of claim 51, wherein the HIV is HIV-1 or HIV-2.

63. A method of reducing the severity of infection of a human infected with HIV, which method comprises administering to the human a severity of infection-reducing amount of an antibody to HIV envelope glycoprotein that can recognize one or more strains of HIV, wherein the epitope of HIV recognized by the antibody is inducible, wherein the antibody binding to the epitope is enhanced by the presence of CD4 and the HIV co-receptor, and wherein the antibody is optionally part of a fusion protein or conjugate, whereupon the severity of the infection of the human is reduced.

20

64. The method of claim 63, wherein the antibody is a neutralizing scFv antibody fragment or an Fab fragment.

65. The method of claim 63, wherein the antibody comprises the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, or a variant of either of the foregoing, wherein the variant retains the ability to bind to the same epitope as that of SEQ ID NO: 1 or SEQ ID NO: 2, respectively, to a greater or lesser extent.

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66. The method of any of claims 63-65, wherein the fusion protein comprises an other antibody that binds to an epitope of HIV.

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67. The method of claim 66, wherein the other antibody that binds to an epitope of HIV is an antibody fragment to HIV envelope glycoprotein that can recognize one or more strains of HIV, wherein the epitope of HIV recognized by the antibody is inducible, and wherein the antibody binding to the epitope is enhanced by the presence of CD4 and the HIV co-receptor.

68. The method of any of claims 63-65, wherein the fusion protein comprises CD4.

69. The method of claim 68, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 7 or a variant thereof, wherein the variant retains the ability to bind to the same epitope as that of SEQ ID NO: 7 to a greater or lesser extent.

70. The method of any of claims 63-65, wherein the fusion protein comprises a toxin.

71. The method of claim 70, wherein the toxin is Pseudomonas toxin.

72. The method of claim 63, wherein the HIV is HIV-1 or HIV-2.

73. A method of treating an infection of a human with HIV, which method comprises administering to the human an isolated or purified nucleic acid molecule encoding an antibody to HIV envelope glycoprotein that can recognize one or more strains of HIV, wherein the epitope of HIV recognized by the antibody is inducible, wherein the antibody binding to the epitope is enhanced by the presence of CD4 and the HIV co-receptor, wherein the antibody is optionally part of a fusion protein, and wherein the isolated or purified nucleic acid molecule is optionally in the form of a vector and/or optionally contained within a cell, whereupon the nucleic acid molecule expresses an infection-treating amount of the antibody in the human and the infection of the human is treated.

74. The method of claim 73, wherein the antibody is a neutralizing scFv antibody fragment or an Fab fragment.

75. The method of claim 73, wherein the isolated or purified nucleic acid molecule encodes the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, or a variant of either of the foregoing, wherein the variant retains the ability to bind to the same epitope as that of SEQ ID NO: 1 or SEQ ID NO: 2, respectively, to a greater or lesser extent, any of which is optionally part of a fusion protein.

76. The method of any of claims 73-75, wherein the fusion protein comprises an other antibody that binds to an epitope of HIV.

77. The method of claim 76, wherein the other antibody that binds to an epitope of HIV is an antibody to HIV envelope glycoprotein that can recognize one or more strains of HIV, wherein the epitope of HIV recognized by the antibody is inducible, and wherein the antibody binding to the epitope is enhanced by the presence of CD4 and the HIV co-receptor.

78. The method of any of claims 73-75, wherein the fusion protein comprises CD4.

79. The method of claim 73, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 8 or a variant thereof, wherein the variant retains the ability to bind to the same epitope as that of SEQ ID NO: 8 to a greater or lesser extent.

80. The method of any of claims 73-75, wherein the fusion protein comprises a toxin.

81. The method of claim 80, wherein the toxin is Pseudomonas toxin.

82. The method of claim 73, wherein the cell is autologous.

83. The method of claim 82, wherein the cell is a B lymphocyte.

84. The method of claim 73, wherein the HIV is HIV-1 or HIV-2.

5 85. A method of treating an infection of a human with HIV, which method comprises administering to the human an infection-treating amount of an antibody to HIV envelope glycoprotein that can recognize one or more strains of HIV, wherein the epitope of HIV recognized by the antibody is inducible, wherein the antibody binding to the epitope is enhanced by the presence of CD4 and the HIV co-receptor, and
10 wherein the antibody is optionally part of a fusion protein or conjugate, whereupon the infection of the human is treated.

 86. The method of claim 85, wherein the antibody is a neutralizing scFv antibody fragment or an Fab fragment.

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 87. The method of claim 85, wherein the antibody comprises the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, or a variant of either of the foregoing, wherein the variant retains the ability to bind to the same epitope as that of SEQ ID NO: 1 or SEQ ID NO: 2, respectively, to a greater or lesser extent.

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 88. The method of any of claims 73-75, wherein the fusion protein comprises an other antibody that binds to an epitope of HIV.

 89. The method of claim 88, wherein the other antibody that binds to an
25 epitope of HIV is an antibody to HIV envelope glycoprotein that can recognize one or more strains of HIV, wherein the epitope of HIV recognized by the antibody is inducible, and wherein the antibody binding to the epitope is enhanced by the presence of CD4 and the HIV co-receptor.

30 90. The method of any of claims 73-75, wherein the fusion protein comprises CD4.

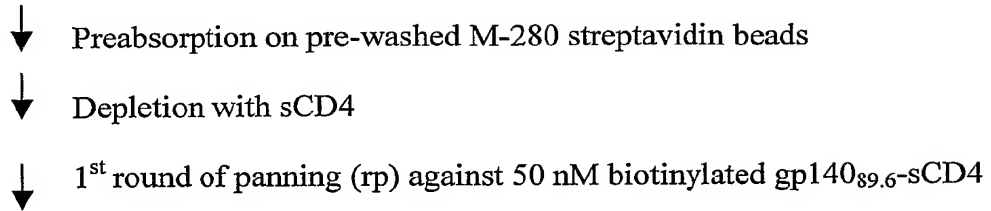
91. The method of claim 90, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 8 or a variant thereof, wherein the variant retains the ability to bind to the same epitope as that of SEQ ID NO: 8 to a greater or lesser extent.

5 92. The method of any of claims 73-75, wherein the fusion protein comprises a toxin.

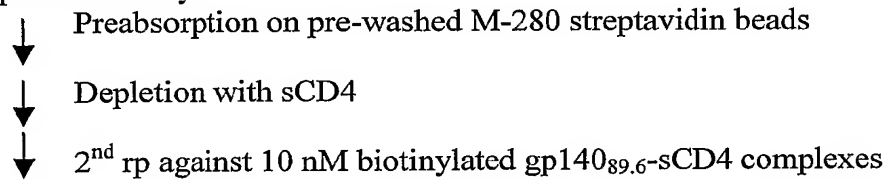
93. The method of claim 92, wherein the toxin is Pseudomonas toxin.

10 94. The method of claim 73, wherein the HIV is HIV-1 or HIV-2.

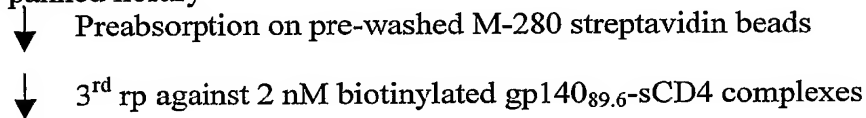
X5 scFv mutagenesis phage library



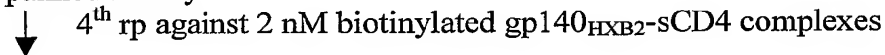
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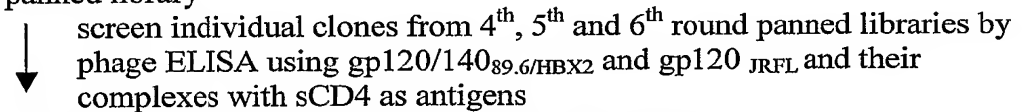
2nd round panned library



3rd round panned library



4th round panned library



selection of clones with highest binding activity to all antigens

FIGURE 1

2/6

	1				50
m9-aa_scFv	VLTQSPGTL	LSAGERATLS	CRASQSVSSG	SLAWYQQKPG	QAPRLLIYGA
X5-aa_scFv	VLTQSPGTL	LSAGERATLS	CRASQSVSSG	SLAWYQQKPG	QAPRLLIYGA
m6-aa_scFv	VLTQSPGTL	LSAGERATLS	CRASQSVSSG	SLAWYQQKPG	QAPRLLIYGA
	51				100
m9-aa_scFv	STRATGIPDR	FSGSGSGTDF	TLTIGRLEPE	DLAVYYCQQY	GTSPYTFGQG
X5-aa_scFv	STRATGIPDR	FSGSGSGTDF	TLTIGRLEPE	DLAVYYCQQY	GTSPYTFGQG
m6-aa_scFv	STRATGIPDR	FSGSGSGTDF	TLTIGRLEPE	DLAVYYCQQY	GTSPYTFGQG
	101				150
m9-aa_scFv	TKLEIKRTGG	GGSGGGGSGG	GGSVQLLEQS	GAEVKKPGSS	VQVSCKASGG
X5-aa_scFv	TKLEIKRTGG	GGSGGGGSGG	GGSVQLLEQS	GAEVKKPGSS	VQVSCKASGG
m6-aa_scFv	TKLEIKRTGG	GGSSGGASGG	GGSVRLLEQS	GAEVKKPGSS	VQVSCKASGG
	151				200
m9-aa_scFv	TFSMYGFNVV	RQAPGHGLEW	MGGIIPIFGT	TNYAQKFRGR	VTFTADQATS
X5-aa_scFv	TFSMYGFNVV	RQAPGHGLEW	MGGIIPIFGT	SNYAQKFRGR	VTFTADQATS
m6-aa_scFv	TFSMYGVNVV	RQAPGHGLEW	MGGIIPIFGT	SNYAQKFRGR	VTFTADQATS
	201				250
m9-aa_scFv	TAYMELTNLR	SDDTAVYYCA	RDFGPDWEGG	DSYDGSGRGF	FDFWGQGTLV
X5-aa_scFv	TAYMELTNLR	SDDTAVYYCA	RDFGPDWEDG	DSYDGSGRGF	FDFWGQGTLV
m6-aa_scFv	TAYMELTNLR	SDDTAVYYCA	RDFGPDWEDG	DSYDGSGRGF	FDFWGQGTLV
	251				
m9-aa_scFv	NVSS	(SEQ ID NO: 1)			
X5-aa_scFv	TVSS	(SEQ ID NO: 3)			
m6-aa_scFv	TVSS	(SEQ ID NO: 2)			

FIGURE 2

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OmpA signal		FR1
<hr/>		
M K K T A I A I A V A L A G F A T V A Q A A E L V L T Q S P		
G T L S L S A		
CDR1		FR2
<hr/>		
G E R A T L S C R A S Q S V S S G S L A W Y Q Q K P G Q A P		
R L L I Y G A		
CDR2	FR3	
<hr/>		
S T R A T G I P D R F S G S G S G T D F T L T I G R L E P E D		
L A V Y Y C Q		
CDR3	FR4	
<hr/>		
Q Y G T S P Y T F G Q G T K L E I K R T V A A P S V F I F P P		
S D E Q L K S		
<hr/>		
G T A S V V C L L N N F Y P R E A K V Q W K V D N A L Q S G		
N S Q E S V		
<hr/>		
T E H D S R D S T Y S L G S T L T L S K A D Y E K H K V Y A		
C E V T H Q G		
<hr/>		
L S S P V T K S F N R G E C (SEQ ID NO: 4)		

FIGURE 3

4/6

PelB Signal										FR1									
M K Y L L P T A A A G L L L L A A Q P A M A E V Q L L E Q S																			
G A E V K K																			
CDR1										FR2									
P G S S V Q V S C K A S G G T F S M Y G F N W V R Q A P G H																			
G L E W M G																			
CDR2										FR3									
G I I P I F G T S N Y A Q K F R G R V T F T A D Q A T S T A Y																			
M E L T N L R																			
CDR3																			
S D D T A V Y Y C A R D F G P D W E D G D S Y D G S G R G																			
F F D F W G Q																			
FR4																			
G T L V T V S S A S T K G P S V F P L A P S S K S T S G G T A																			
A L G C L V																			
K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S																			
S G L Y S L S																			
S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K																			
K V E P K S C																			
D K T S (SEQ ID NO: 5)																			

FIGURE 4

5/6

atgaaccggggagtccttttaggcacttgcttctggtgctgcaactggcgctcctcccagcagccactcagggaaagaaagt
ggtgctgggcaaaaaaggggatacagtgggaactgacctgtacagcttccagaagaagagcatacaattccactggaaaaa
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gggaagaccctctccgtgtctcagctggagctccaggatagtggcacctggacatgcactgtcttcagaaccagaagaag
gtggagttcaaaatagacatcgtggtgctagct (SEQ ID NO: 6)

1 MNRGVPFRLH LLVLQLALLP AATQGKKVVL GKKGDTVELT
CTASQKKSIQ
51 FHWKNSNQIK ILGNQGSFLT KGPSKLNDRA DSRRLWDQG NFPLIKNLK
101 IEDSDTYICE VEDQKEEVQL LVFGLTANS DTHLLQGQSLT LTLESPPGSS
151 PSVQCRSPRG KNIQGGKTLS VSQLELQDSG TWTCTVLQNN
KKVEFKIDIV
201 VLA (SEQ ID NO: 7)

FIGURE 5

VLTQSPGTL SLSAGERATL SCRASQSVSSGSLAWYQQKPGQAPRLLIYGASTRA
TGIPDRFSGSGSGTDFTLTIGRLEPEDLAVYYCQQYGTSPYTFGQGTKLEIKRTG
GGGSSGGASGGGGSVRLLEQSGAEVKKPGSSVQVSCKASGGTFSMYGVNWV
RQAPGHGLEWMGGIPIFGTSNYAQKFRGRVTFTADQATSTAYMELTNLRSDD
TAVYYCARDFGPDWEDGDSYDGSGRGFFDFWGQGT LVT VSS **GGGGSGGG**
GSGGGGSGGGGSGGGGSGGGGSMNRGV PFRHLLVLQLALLPAATQ
GKKVVLGKKGDTVELTCTASQKKSIQFWKNSNQIKILGNQGSFLT KGPSKLN
DRADSRRLWDQGNFPLIKNLKIEDSDTYICEVEDQKEEVQLLVFGLTANSDT
HLLQGQSLT LTLESPPGSSPSVQCRSPRGKNIQGGKTL SVSQLELQDSGTWTCT
VLQNQKKVEFKIDIVVLA (SEQ ID NO: 8)

FIGURE 6

SEQUENCE LISTING

<110> THE GOVERNMENT OF THE UNITED STATES OF AMERICA, REPRESENTED
BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES

<120> IDENTIFICATION OF NOVEL BROADLY CROSS-REACTIVE HIV-1 NEUTRALIZING
HUMAN MONOCLONAL ANTIBODIES

<130> 222179

<150> 60/378,406

<151> 2002-05-06

<160> 8

<170> PatentIn version 3.2

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35 40 45

Gly Ala Ser Thr Arg Ala Thr Gly Ile Pro Asp Arg Phe Ser Gly Ser
50 55 60

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Gly Arg Leu Glu Pro Glu
65 70 75 80

Asp Leu Ala Val Tyr Tyr Cys Gln Gln Tyr Gly Thr Ser Pro Tyr Thr
85 90 95

Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys Arg Thr Gly Gly Gly Gly
100 105 110

Ser Gly Gly Gly Gly Ser Gly Gly Gly Ser Val Gln Leu Leu Glu
115 120 125

Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser Ser Val Gln Val Ser
130 135 140

Cys Lys Ala Ser Gly Gly Thr Phe Ser Met Tyr Gly Phe Asn Trp Val
145 150 155 160

Arg Gln Ala Pro Gly His Gly Leu Glu Trp Met Gly Gly Ile Ile Pro
165 170 175

Ile Phe Gly Thr Thr Asn Tyr Ala Gln Lys Phe Arg Gly Arg Val Thr
180 185 190

Phe Thr Ala Asp Gln Ala Thr Ser Thr Ala Tyr Met Glu Leu Thr Asn
195 200 205

Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys Ala Arg Asp Phe Gly
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Pro Asp Trp Glu Gly Gly Asp Ser Tyr Asp Gly Ser Gly Arg Gly Phe
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Gly Ala Ser Thr Arg Ala Thr Gly Ile Pro Asp Arg Phe Ser Gly Ser
50 55 60

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Gly Arg Leu Glu Pro Glu
65 70 75 80

Asp Leu Ala Val Tyr Tyr Cys Gln Gln Tyr Gly Thr Ser Pro Tyr Thr
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Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys Arg Thr Gly Gly Gly Gly
Page 2

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 130 135 140
 Cys Lys Ala Ser Gly Gly Thr Phe Ser Met Tyr Gly Val Asn Trp Val
 145 150 155 160
 Arg Gln Ala Pro Gly His Gly Leu Glu Trp Met Gly Gly Ile Ile Pro
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 Ile Phe Gly Thr Ser Asn Tyr Ala Gln Lys Phe Arg Gly Arg Val Thr
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 Gly Ala Ser Thr Arg Ala Thr Gly Ile Pro Asp Arg Phe Ser Gly Ser
 50 55 60

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 Ile Phe Gly Thr Ser Asn Tyr Ala Gln Lys Phe Arg Gly Arg Val Thr
 180 185 190
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 Ile Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu
 85 90 95
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 Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn
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 435 440 445
 Gly Gly Lys Thr Leu Ser Val Ser Gln Leu Glu Leu Gln Asp Ser Gly
 450 455 460
 Thr Trp Thr Cys Thr Val Leu Gln Asn Gln Lys Lys Val Glu Phe Lys
 465 470 475 480
 Ile Asp Ile Val Val Leu Ala
 485

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/14095

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07K 16/00; A61K 39/21, 31/70; C07H 21/04
US CL : 530/388.35; 424/188.1, 208.1; 536/23.4; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/388.35; 424/188.1, 208.1; 536/23.4; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
USPATFUL, WPIDS, EUROPATFUL, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	MOULARD, M. et al. Broadly cross-reactive HIV-1-neutralizing human monoclonal Fab selected for binding to gp120-CD4-CCR5 complexes. PNAS. 14 May 2002, Vol. 99, No. 10, pages 6913-6918, see entire document.	1-28
Y, P		29-94
Y, P	FINNEGAN, C. F. et al. Antigenic properties of the human immunodeficiency virus transmembrane glycoprotein during cell-cell fusion. Journal of Virology. December 2002, Vol. 76, No. 23, pages 12123-12134, see entire document.	1-94

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T"

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"

document member of the same patent family

Date of the actual completion of the international search

25 August 2003 (25.08.2003)

Date of mailing of the international search report

10 SEP 2003

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Facsimile No. (703)305-3230

Authorized officer

I/S. Parkin

Telephone No. (703) 308-0196